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## Contents



NATIONAL CANCER INSTITUTE
NATIONAL INSTITUTES OF HEALTH
DEPARTMENT OF HEALTH AND HUMAN SERVICES

■ TRANSLATIONAL RESEARCH

# DNA Double-strand Breaks and Aging

Sedelnikova OA, Horikawa I, Zimonjic DB, Popescu NC, Bonner WM, and Barrett JC. Senescing human cells and ageing mice accumulate DNA lesions with unrepairable double-strand breaks. *Nat Cell Biol* 6: 168–70, 2004.

Humans and animals have a limited lifespan, and cell cultures derived from them cease proliferating and enter senescence after a finite number of population doublings. Oxidative stress, DNA damage, and in humans, telomere shortening are all suggested as major factors in aging at cellular levels. However, how well cellular senescence in *in vitro* models corresponds to aging *in vivo* is unclear. Our study aims at understanding the relationship between cellular and organismal aging in mammals.

Several findings have supported a role for DNA damage/repair in aging. Mice deficient in DNA repair proteins exhibit aging phenotypes earlier than wild-type mice (Celeste A et al. *Science* 296: 922–7, 2002). In addition, several DNA damaging agents induce a cellular senescence-like state in culture without extensive cell division (premature senescence) (Horikawa I et al. *J Anti Aging Med* 3: 373–82, 2000). To obtain more direct evidence for a direct link between DNA damage and mammalian aging, we immunostained cultures of normal human cells at different population doublings and touch prints of tissues from mice of different ages for foci of γ-H2AX, the phosphorylated form of histone H2AX that specifically marks DNA double-strand breaks (DSBs) (Rogakou EP. *J Cell Biol* 146: 905–16, 1999; Sedelnikova OA et al. *Radiat Res* 158: 486–92, 2002).

DNA DSBs were revealed in both experimental systems as γ-H2AX foci (γ-foci). In addition, the incidences of γ-foci were found to increase with senescence *in vitro* and age *in vivo* as measured by counting γ-foci in individual nuclei. In three normal human cell strains, the number of γ-foci increased with replicative aging from 0.1 to 0.3 foci per young cell to 2.2 to 4.1 foci per senescent cell. Fibroblastic and epithelial cells exhibited similar increases. Cultures subjected to premature senescence with the DSB inducer

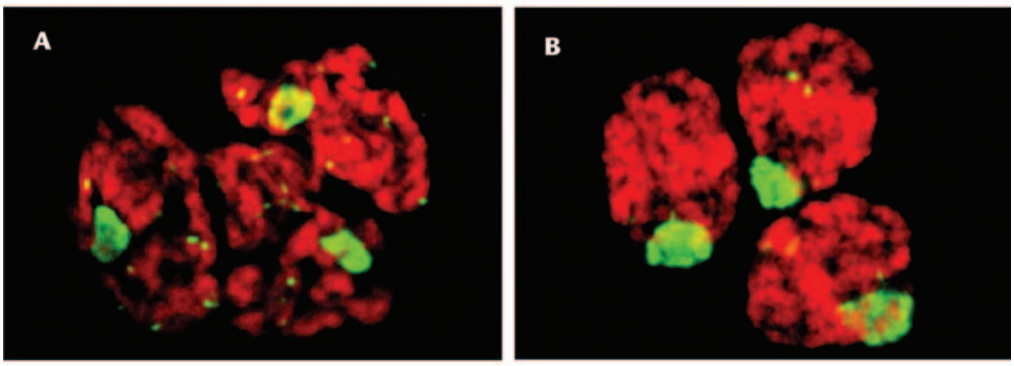


**Figure 1.** Confocal microscopy image showing accumulation of γ-H2AX signal in nuclei of testes cells from an old mouse (*A*) compared with a young mouse (*B*). The tissues were touch printed and processed for immunocytochemistry. Large green spots are pachytene spermatides with ongoing homologous recombination.

bleomycin and the oxidative stress inducer hydrogen-peroxide also exhibited increased incidences of γ-foci characteristic of more senescent phenotypes.

Studies with mice yielded similar findings (Figure 1). Touch-print immunostaining of cells from the liver, kidney, lung, brain, and testes taken from mice of different ages revealed increased incidences of γ-foci during *in vivo* aging similar to those found *in vitro*. Thus, the accumulation of γ-foci is a common process in mammalian aging *in vivo* and in culture.

These foci of unknown origin are named cryptogenic foci to differentiate them from those of known causes such as ionizing radiation. When cell cultures or mice were irradiated with 0.6 Gy, we observed the expected increases in the incidences of γ-foci 30 minutes post-irradiation, followed by the expected slower decreases, until at 24 hours post-irradiation, the incidences of γ-foci returned to values near the pre-irradiation values characteristic of the stage of senescence or age of the treated cells. Importantly, this finding indicates that the cryptogenic foci are stable before, during, and after the induction and disappearance of those induced by ionizing radiation. Thus, it is likely that the cryptogenic foci mark persistent and unrepairable DNA lesions.

Cryptogenic foci accumulate the DNA repair proteins, 53BP1, Mre11, Rad50, and Nbs1, indicating that they are sites of ongoing or attempted DNA repair. In addition, since repair proteins accumulate at γ-foci with different kinetics, it may be possible to differentiate cryptogenic foci and nascent foci induced by ionizing radiation by their content of DNA repair factors soon after irradiation. We observed that the accumulation of DNA repair proteins is incomplete on a subset of foci at 30 minutes post-irradiation but complete on all at 60 minutes, indicating that DNA repair factors were already present at the cryptogenic foci but in the process of accumulation at the nascent foci. Thus, this observation supports a model of two types of γ-H2AX foci: (1) transient, where successful DSB rejoining occurs, and (2) persistent, containing unrepairable DSBs. On the other hand, cryptogenic foci do not colocalize with telomeres to a significant extent, suggesting that the senescence/aging-associated DSBs in cryptogenic foci are primarily at non-telomeric sites.

This study demonstrates that during cellular senescence or organismal aging, mammalian cells accumulate persistent DNA lesions that contain unrepairable DSBs. The similar incidence of persistent γ-H2AX foci associated with cellular senescence, either prematurely induced by the exogenous agents or after cell divisions, supports a model in which the accumulation of unrepairable DSB-containing lesions may play a causal role in aging. Hence, this study establishes the physiological importance of unrepairable DSB-containing lesions in cellular and organismal aging in mammals, and raises the possibility that diverse factors that affect aging may all act ultimately through the accumulation of persistent DNA lesions containing unrepairable DSBs. One area of future interest is to determine whether γ-foci accumulate at specific chromosomal sites or structures. Our study also suggests that γ-foci may be useful markers for detecting individual senescent cells in aged mammals. We believe that our findings will lead to a better understanding of how cellular senescence contributes to organismal aging and how aging can be a major risk factor in human carcinogenesis.


- **Olga Sedelnikova, PhD**
  Staff Scientist
  Laboratory of Molecular Pharmacology
  NCI-Bethesda, Bldg. 37/Rm. 5050
  Tel: 301-402-3649
  Fax: 301-402-0752
  olgas@helix.nih.gov
- **Izumi Horikawa, PhD**
  Staff Scientist
  Laboratory of Biosystems and Cancer
  NCI-Bethesda, Bldg. 37/Rm. 5046
  Tel: 301-594-8510
  Fax: 301-480-2772
  horikawi@mail.nih.gov


## BIOTECHNOLOGY RESOURCES

# Immunological Monitoring of Cancer Vaccine Trials

Monitoring T-cell responses in the course of clinical trials is widely used to aid in assessing the efficacy of cancer immunotherapy. The clinical investigator is faced with selecting an *ex vivo* monitoring method that provides the best measure of immune reactivity in order to determine any correlation between clinical and immunologic responses to specific immunotherapy. The selection and application of appropriate assays are challenges best met by utilizing core laboratories with extensive experience in performing immunological assays to monitor clinical trials. The Laboratory of Cell-Mediated Immunity (LCMI) is just such a core laboratory, providing this service to NIH researchers. LCMI is part of the Clinical Services Program, SAIC-Frederick, Inc., located on the NCI-Frederick campus. The overall mission of the laboratory is to develop and provide state-of-the-art, clinically validated, immunological assays that are of broad applicability to the investigators within NIH. Moreover, LCMI serves as an independent laboratory for monitoring clinical trials. The laboratory is composed of highly trained personnel who have expertise in the development, execution, and analysis of complex immunological assays.

Validated assays currently available for clinical monitoring of human samples

include the peptide IFN (interferon)-γ ELISPOT, whole protein IFN-γ ELISPOT, autologous tumor IFN-γ ELISPOT, Granzyme B (GrB) ELISPOT, cell proliferation, cytokine induction, CTL (cytotoxic T lymphocyte) induction, and $^{51}$Cr (chromium)-release. LCMI also has optimized the ELISPOT assay for detection of IFN-γ secretion in the murine system. All assays have been approved for use by the Vaccine Working Group Steering Committee (Chair Dr. Jay Berzofsky) and are available to NIH researchers. The combination of these assays provides valuable information for immunological monitoring of cancer vaccine trials.

The standard immunological assays, such as cytokine induction, cell proliferation, and $^{51}$Cr-release, can detect immune responses in vaccinated patients but are not suitable for evaluation of individual cell reactivity. In contrast, the ELISPOT assay can measure the frequency of reactive cells and is more sensitive. The ELISPOT assay, a modification of the ELISA, utilizes antibody-coated membranes to detect locally secreted cytokines or other immune proteins by individual cells. The release of immune proteins from activated cells results in spot formation. At appropriate cellular concentrations, each spot formed represents a single reactive cell. Thus, the ELISPOT assay provides both qualitative (type of immune protein) and quantitative (number of responding cells) information. The assay has gained increasing popularity, especially as a surrogate measure for CTL responses, because it is both reliable and highly sensitive.

The peptide IFN-γ ELISPOT is suitable for monitoring T-cell reactivity to HLA-A2–binding, 9- or 10-mer peptides. A whole-protein IFN-γ ELISPOT was developed by LCMI to monitor immune responses to vaccinations with whole proteins or peptides greater than 10 mer. To determine the optimal system for individual antigens, the effects of anti-CD28 antibodies (anti-CD28) versus dendritic cells (DCs) versus anti-CD28 and DCs have been compared with peripheral blood mononuclear cells (PBMCs) plus antigen alone. Results have shown variation in the level of response between different antigens and systems; therefore, the assay should be individually optimized.

The ELISPOT assay has been primarily used for the detection of T-cell responses following vaccination, by using peptide or protein pulsed antigen-presenting cells as surrogate T-cell targets. However, the reactivity to vaccine components does not necessarily equate to recognition and elimination of tumor cells. An alternative approach is to test the direct reactivity of T cells against autologous tumor cells. Therefore, LCMI has developed and validated an autologous tumor IFN-γ ELISPOT assay. This assay can be directly applied when patient tumor cells are available, tumor-specific antigens have not been fully identified, or when whole or lysed tumor cells are used as the immunogen.

One of the most promising new assays available to NIH investigators is the GrB ELISPOT assay. GrB is the most abundant granzyme present in cytolytic granules of CTL and natural killer (NK) cells and is a key mediator of target cell death. Therefore, the release of GrB may be used to measure NK and CTL cytotoxicity. Numerous comparison studies between the GrB ELISPOT and $^{51}$Cr-release assays were performed at the LCMI, and the lab's findings demonstrate that the GrB ELISPOT is a superior alternative to the $^{51}$Cr-release assay since it is more sensitive and uses fewer effector cells. Moreover, unlike the IFN-γ ELISPOT assay, which is a surrogate marker of killing, the GrB ELISPOT directly measures cytotoxic cell activity.

Modifications of the ELISPOT assays developed by LCMI allow for more comprehensive evaluation of low-frequency tumor-specific CTLs and their effector functions and can provide valuable insight with regard to immune responses in cancer vaccine trials. On a regular basis, LCMI evaluates and adapts different ELISPOT vendor kits, reagents, antibodies, plates, and spot visualization systems to enhance the ELISPOT assays. Assay systems are routinely quality controlled.

All ELISPOT assays are subjected to automated analysis using the Immunospot Analyzer from Cellular Technology, Ltd. The laboratory continues to tailor other current methods as well as develop new procedures to assess immune responses in clinical trials.

To contact LCMI for immunological testing, please contact Dr. Anatoli Malyguine (amalyguine@ncifcrf.gov, 301-846-1890) or Susan Strobl (sstrobl@ncifcrf.gov, 301-846-6922). To formally request LCMI services, please go to the NCI Yellow Task web site at http://web.ncifcrf.gov/campus/yellowtask.


- **Anatoli Malyguine, MD, PhD**
- **Susan Strobl**
- **Kimberly Shafer-Weaver**

Laboratory of Cell-Mediated Immunity
Clinical Services Program
SAIC-Frederick, Inc.
NCI-Frederick, Bldg. 560/Rm. 12-79
Tel: 301-846-1890
Fax: 301-846-6757
amalyguine@ncifcrf.gov

# The Perils of Abrogating *Myc*-dependent Apoptosis

Cheung WC, Kim JS, Linden M, Peng L, Van Ness B, Polakiewicz RD, and Janz S. Novel targeted deregulation of c-Myc cooperates with Bcl-X(L) to cause plasma cell neoplasms in mice. *J Clin Invest* 113: 1763–73, 2004.

A new paradigm is emerging in cancer biology owing to the growing realization that oncogenes, which were once believed to merely provide a growth advantage to incipient cancer cells, can paradoxically also put these cells at grave risk of suicide by apoptosis. The master regulator *Myc* is the quintessential oncogene in this regard. Although *Myc* is required in normal and malignant cells for cell growth and cell cycle progression, it effectively kills cells when it is overexpressed or deregulated in stressed environments. *Myc*-dependent pathways that trigger the apoptotic response have come to be understood as important checkpoints that delete cells that had undergone *Myc*-induced neoplastic transformation. Not surprisingly, these pathways are often selectively bypassed during tumor development.

More than 20 years ago, a series of studies in the laboratories of Michael Potter, MD, and Frederic Mushinski, MD, at NCI's Laboratory of Genetics, and by others around the world, established that peritoneal mouse plasmacytomas, neoplasms of immunoglobulin-producing plasma cells, are induced by reciprocal chromosomal translocations that result in the deregulated expression of *Myc*. The most common *Myc*-activating translocation (~90%) is the T(12;15), which joins the *Myc* gene to the immunoglobulin heavy-chain locus, *Igh*, juxtaposing *Myc*, in approximately 85% of cases, to the most downstream *Igh* constant gene, *Cα*. Following chromosomal translocation, Myc expression is driven by the powerful *Igh* 3′-*Cα* enhancer, which—in accordance with its physiological function of enhancing immunoglobulin heavy-chain expression in plasma cells—reaches peak activity in plasma cells.

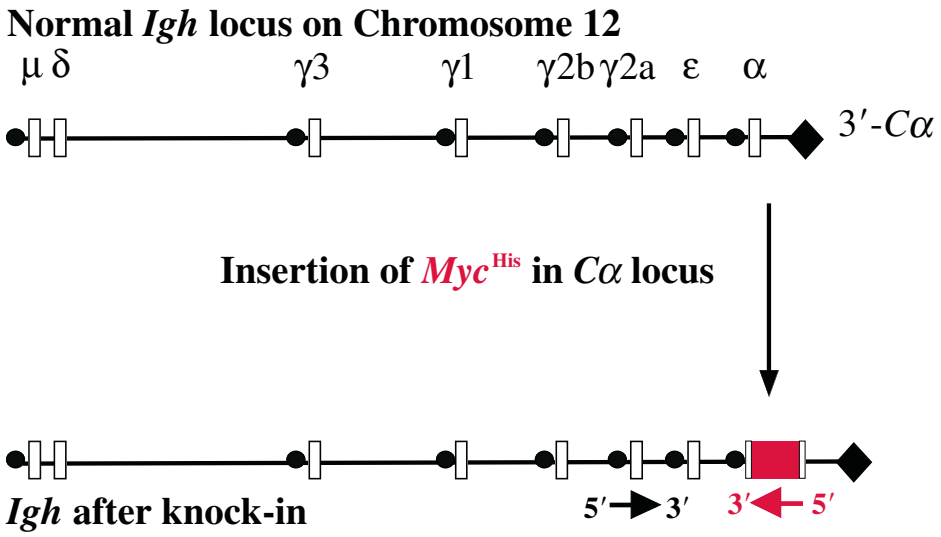


**Figure 1.** Generation of iMyc$^{Cα}$ transgenic mice. Shown are the normal mouse *Igh* locus (top) and the targeted *Igh* locus with the inserted *Myc*$^{His}$ gene (bottom). The transcriptional orientations of *Igh* and *Myc*$^{His}$ are indicated by black and red arrows, respectively. The 3′-*Cα* enhancer is depicted as a black diamond. This is the first time gene insertion in mice has been used to reproduce an oncogene-activating chromosomal translocation of great relevance to human cancer.

We hypothesized that mimicking the *Myc-Cα* juxtaposition by gene insertion in mice might result in a good model of T(12;15) translocation and, thereby, recapitulate the mode of *Myc* deregulation that is conducive to plasmacytoma development. In collaboration with Lino Tessarollo, PhD, from NCI's Mouse Cancer Genetics Program, we generated *Myc* transgenics that harbor a single-copy histidine-tagged mouse *Myc* gene, *Myc*$^{His}$, inserted head-to-head into the mouse *Cα* locus. We refer to these mice as iMyc$^{Cα}$ (Figure 1). Somewhat disappointingly, the iMyc$^{Cα}$ mice developed plasma cell tumors infrequently and only after a long latency. This suggested that although the *Myc* transgene reproduced the requisite molecular changes that initiate neoplastic plasma cell development in mice, *Myc*'s true oncogenic potential *in vivo* was tempered, possibly by *Myc*-dependent apoptosis.

*Myc-dependent pathways that trigger the apoptotic response have come to be understood as important checkpoints that delete cells that had undergone Myc-induced neoplastic transformation.*

To test this possibility, we crossed the *Myc* transgenic mice with *Bcl2l1* (Bcl-$X_L$) transgenic mice that were recently developed in the laboratory of Brian Van Ness, PhD at the University of Minnesota. In this mouse strain, the expression of the death suppressor *Bcl2l1* is driven by the mouse immunoglobulin κ light-chain 3′enhancer, which exhibits, just like the *Igh* 3′-*Cα* enhancer, peak activity in plasma cells. Subsequent studies with the research group of Roberto Polakiewicz, PhD at Cell Signaling Technology, Inc., Beverly, MA, showed that similar to the iMyc$^{Cα}$ mice, single transgenic *Bcl2l1* mice demonstrated a weak tumor phenotype. In contrast, the double transgenic

*Myc-Bcl2l1* mice developed plasma cell tumors with short onset (135 days on average) and full penetrance (100% tumor incidence). These tumors produced monoclonal immunoglobulin, infiltrated the bone marrow, and caused in some cases, osteolytic lesions leading to pathological bone fractures.

Deregulated expression of *MYC* and death suppressor genes of the *BCL2* family, such as *BCL2L1* and *MCL1*, is a consistent feature of human plasma cell neoplasms, including multiple myeloma, which comprises the second most common blood cancer in the United States. Multiple myeloma is further characterized by osteolytic bone lesions and pathological fractures. Our studies showed that the enforced expression of *Myc* and *Bcl2l1* by immunoglobulin enhancers with peak activity in plasma cells generates a mouse model of human multiple myeloma that may be useful in elucidating the mechanism of the *Myc-Bcl2l1* collaboration and in designing new approaches for treatment and prevention of human multiple myeloma.

■ **Siegfried Janz, MD**
Principal Investigator
Laboratory of Genetics
NCI-Bethesda, Bldg. 37/Rm. 3140A
Tel: 301-496-2202
Fax: 301-402-1031
sj4s@nih.gov

## ■ CHEMISTRY

# The Targeting of Ascorbate by Nickel and Cobalt

Salnikow K, Donald SP, Bruick RK, Zhitkovich A, Phang JM, and Kasprzak KS. Depletion of intracellular ascorbate by the carcinogenic metals nickel and cobalt results in the induction of hypoxic stress. *J Biol Chem* 279: 40337–44, 2004.

Nickel and cobalt have wide industrial usage, which eventually leads to environmental pollution by these metals and their byproducts at all stages of production, recycling, and disposal. They also are present in fossil fuels, and the burning of fossil fuels pollutes the air with metal-containing particles. Environmental or occupational exposure to particles containing nickel or cobalt causes various forms of lung injury including pneumonitis, asthma, and fibrosis. Both metals are carcinogenic in animals, and nickel has been recognized as a human carcinogen. The mechanisms of toxic and carcinogenic activity of these metals are not fully understood. An interesting feature of nickel or cobalt exposure is the induction of hypoxia-like stress, which is manifested in cells by the activation of the hypoxia-inducible factor-1 (HIF-1) transcription factor and hypoxia-inducible genes.

The HIF-1 transcription factor is a heterodimer composed of α and β subunits. The activity of HIF-1 depends on the accumulation of short-lived HIFα. Under normoxic conditions, hydroxylation of proline residues 402 and 564 (Pro-402/564) in the oxygen-dependent degradation domain (ODD) of HIF-1α leads to HIF-1α's interaction with the von Hippel-Lindau (VHL) tumor-suppressor protein (a part of the ubiquitin-ligase complex), followed by its ubiquitylation and rapid proteosomal degradation. Under hypoxic conditions, limiting oxygen decreases hydroxylation, which prevents VHL binding and leads to the accumulation of HIF-1α protein. Recent studies indicate that the family of iron-containing dioxygenases that hydroxylate prolines in HIF-1α (called prolyl hydroxylase domain proteins [PHDs]) plays an "oxygen sensor" role. It has been suggested that the induction of the hypoxia-like stress by nickel or cobalt is based on the ability of these metals to take the place of an iron atom in an "oxygen sensor." No direct evidence supporting this hypothesis is available, although several studies have demonstrated that cobalt can inhibit the activity of recombinant hydroxylase *in vitro*.

The purpose of our study was to investigate the effect of metals on the "oxygen sensor" (i.e., PHD) and the hydroxylation of Pro-402/564 of HIF-1α. Indeed, metals added to fractionated cell extracts partially inhibited ODD-dependent enzymatic activity, but we suggested that this was not enough to completely prevent HIF-1α hydroxylation.

Next we investigated whether the hydroxylation of Pro-402/564 is affected by nickel or cobalt in living cells. To test this, we designed a plasmid expressing a recombinant reporter protein containing the ODD fragment of HIF-1α fused with luciferase (CMV-Luc-ODD). It was expected that, similar to the HIF-1α protein, the half-life of CMV-Luc-ODD would depend on the hydroxylation status of Pro-402/564. To test this, the CMV-Luc-ODD reporter was transfected into 786-0 renal carcinoma cells lacking functional VHL and into the same cells that were stably transfected with normal VHL. The experiments showed that the Pro-402/564 of the reporter were normally hydroxylated in human lung epithelial cells, allowing the recombinant protein to interact with VHL followed by its degradation, similar to HIF-1α protein. Exposure to cobalt or nickel prevented proline hydroxylation and stabilized reporter protein.

Prolyl hydroxylases are non-heme iron-dependent dioxygenases, which utilize 2-oxoglutarate (2OG) as a co-substrate and ascorbic acid as a co-factor. During the hydroxylation reaction, the molecular oxygen is split into two atoms, one of which is converted to the hydroxyl group attached to an amino acid. The other one is incorporated into $CO_2$, following decarboxylation of 2OG. Prolyl hydroxylase can run a few cycles without iron oxidation, but eventually the enzyme-bound iron(II) is converted to iron(III), which causes the inactivation of the enzyme. The role of ascorbate is to reduce iron(III) and thus reactivate the enzyme. This scenario implies that the depletion of ascorbate must lead to the

loss of enzymatic activity. Therefore, we further investigated the effect of metals on intracellular levels of ascorbate and ascorbate uptake by cells. We demonstrated that the intracellular ascorbate was greatly depleted by exposure to both metals, and the addition of ascorbate restored the intracellular level of ascorbate and the hydroxylation of the reporter protein. The addition of ascorbate also inhibited expression of endogenous hypoxia-inducible genes upregulated by nickel or cobalt.

The finding that metals strongly deplete intracellular ascorbate and inhibit cellular hydroxylases has profound implications for prevention of metal-induced lung injuries. It explains the effect of metals on hypoxia-like stress as well as impairment of protein maturation dependent on hydroxyproline. The hydroxyproline-containing proteins include collagens, an important part of extracellular matrix, surfactant, and complement proteins. Damaging surfactant A and C and complement C1q proteins following inhalation of metal-containing particles undoubtedly can lead to a variety of respiratory diseases (e.g., pneumonitis, asthma, and fibrosis). Moreover, recently discovered DNA dioxygenases that repair alkylated modifications of DNA bases can be inactivated by ascorbate depletion. Hypoxia is widespread in tumors. It upregulates HIF-1 transcription factor and the expression of hypoxia-inducible genes, which is a typical feature of transformed phenotype. Thus, the depletion of intracellular ascorbate by metals produces hypoxia-like stress, which may establish intracellular and extracellular conditions for the selection of cells with a transformed phenotype.


- **Konstantin Salnikow, PhD**
  Staff Scientist
  salnikow@ncifcrf.gov
- **Kazimierz Kasprzak, PhD, DSc**
  Principal Investigator
  Laboratory of Comparative Carcinogenesis
  NCI-Frederick, Bldg. 538/Rm. 205E
  Tel: 301-846-5738
  Fax: 301-846-5946
  kasprkaz@ncifcrf.gov


■ TUMOR BIOLOGY

# Searching for Metastatic Regulators

Yu Y, Khan J, Khanna C, Helman L, Meltzer PS, and Merlino G. Expression profiling identifies the cytoskeletal organizer ezrin and the developmental homeoprotein Six-1 as key metastatic regulators. *Nat Med* 10: 175–81, 2004.

We have been studying the metastasis of the skeletal muscle cancer rhabdomyosarcoma (RMS), which accounts for nearly 10% of all pediatric neoplasms and for more than half of pediatric soft tissue sarcomas. Approximately one third of patients with RMS experience relapses, and the majority of these patients die from disseminated metastatic disease, illustrating the need for new therapeutic strategies. The development of cancer therapeutics can benefit enormously when relevant animal models that accurately reflect human disease are available. Previously, we described a genetically engineered mouse in which constitutive activation of the receptor tyrosine kinase c-Met and loss of function at the tumor suppressor locus *ink4a/arf* induced skeletal muscle tumors reminiscent of human RMS with high penetrance and short latency (Sharp R et al. *Nat Med* 8: 1276–80, 2002). Recently, we established a panel of highly and poorly metastatic RMS cell lines from these primary RMS tumors, which were subjected to global microarray-based expression profiling to identify genes that might contribute to the metastatic process. Figure 1 depicts the general approach used and the experimental flow of this study.

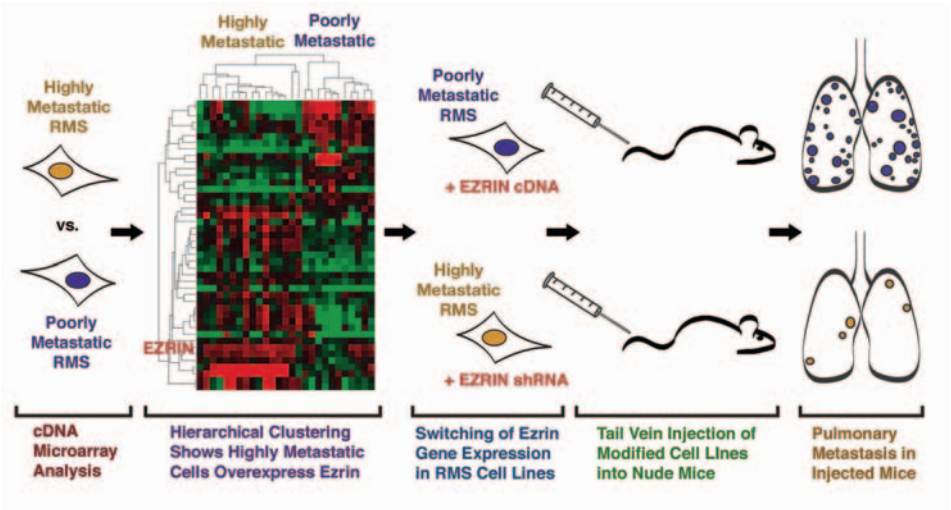


**Figure 1.** Identifying and validating novel metastasis regulators. Highly and poorly metastatic cell lines were established from a genetically engineered mouse model of rhabdomyosarcoma (RMS). Microarray-based expression profiling of RNA isolated from these lines was then used to identify genes whose expression could potentially dictate metastatic potential. (Red boxes denote high expression, green low.) *Ezrin* is shown as an example of a gene characteristically overexpressed in highly metastatic cell lines. Switching the expression of individual candidate genes in representative rhabdomyosarcoma cell lines validated their metastatic role. For example, *ezrin* activity was genetically enhanced in poorly metastatic lines (through forced expression of *ezrin* cDNA), and suppressed in highly metastatic lines (through forced expression of *ezrin* small hairpin RNA [shRNA] or a dominant-negative ezrin). The resulting modified lines were introduced into nude mice by tail vein injection, and the number of resulting pulmonary metastases provided readout for changes in metastatic potential.

Random permutation-weighted gene analysis was used to identify the most significant differentially expressed genes that distinguish the highly from the poorly metastatic lines. Based on this set of genes, the highly and poorly metastatic RMS cell lines clustered into two separate classes (Figure 1). After confirming that these microarray data accurately represented the true gene expression patterns within the RMS cells, we focused on two genes that were characteristically overexpressed in highly metastatic RMS cells: *ezrin* and *Six-1*.

Ezrin protein provides a functional link between the cellular cytoskeleton and the plasma membrane and resides at the nexus of pathways regulating cell survival, motility, invasion, and adherence. The effect of ezrin on metastasis was experimentally plumbed by inserting an ezrin-expression vector into poorly metastatic, low-ezrin–expressing cell lines, and injecting the resulting modified RMS cells into the tail vein of nude mice (Figure 1). Ezrin expression significantly stimulated pulmonary metastasis in poorly metastatic cell lines. Conversely, when ezrin activity was disrupted in highly metastatic RMS cell lines through expression of either a dominant-negative ezrin molecule, or a small hairpin RNA (shRNA), metastatic potential dropped significantly. Together, these data indicate that ezrin is an important determinant of metastatic potential in RMS cells and may be required for efficient metastasis.

Six-1, a vertebrate homolog of the *Drosophila sine oculis* gene product, is a homeodomain-containing transcription factor required for skeletal muscle development. Using the same experimental approach, the role of Six-1 in metastasis was examined by placing a Six-1 expression vector into poorly metastatic RMS cell lines characterized by low Six-1 expression. As with ezrin, elevated Six-1 expression significantly enhanced the metastatic potential of RMS cell lines. Conversely, expression of Six-1–specific shRNA in highly metastatic RMS cells reduced Six-1 expression and significantly inhibited pulmonary metastasis. These data show that the status of Six-1 expression can also profoundly affect metastatic potential.

The ezrin and Six-1 data tell us much about the importance of these molecules in murine metastasis. However, are the findings relevant to human disease? To answer this question, we quantified EZRIN and SIX1 expression in human samples of stage 1, 2, and 4 RMS. Notably, EZRIN and SIX1 were both found to be overexpressed in human RMS, with expression levels significantly correlating with tumor progression. EZRIN has also recently been shown to play a critical role in osteosarcoma metastasis (Khanna C et al. *Nat Med* 10: 182–6, 2004).

Thus, microarray-based genomic analysis can be combined with genetically engineered mouse modeling to dissect metastatic mechanisms. The successful identification and functional confirmation of ezrin and Six-1 as critical metastatic regulators offers new mechanistic and perhaps therapeutic insights into RMS. Both *ezrin* and *Six-1* are members of a panel of genes whose predictive value with respect to metastatic potential can now be validated both prospectively in the mouse, as well as retrospectively in patients. Ezrin may also prove valuable as a new molecular target of anti-metastasis therapy.


■ **Yanlin Yu, PhD**
Research Fellow
Laboratory of Cell Regulation and Carcinogenesis
NCI-Bethesda, Bldg. 37/Rm. 5002
Tel: 301-402-4073
Fax: 301-480-7618
YuY@mail.nih.gov

■ **Glenn Merlino, PhD**
Chief
Laboratory of Cell Regulation and Carcinogenesis
NCI-Bethesda, Bldg. 37/Rm. 5002
Tel: 301-496-4270
Fax: 301-480-7618
gmerlino@helix.nih.gov


■ CELL BIOLOGY

# Relaying Attractant Signals During Chemotaxis

Kriebel PW, Barr VA, and Parent CA. Adenylyl cyclase localization regulates streaming during chemotaxis. *Cell* 112: 549–60, 2003.

A wide variety of cells possess the ability to sense and migrate up gradients of chemoattractants. This behavior, referred to as *chemotaxis*, is important for a variety of physiological and pathological processes including axon guidance, angiogenesis, wound healing, and metastasis. Leukocytes homing to sites of inflammation perhaps represent the quintessential example of this behavior. When exposed to chemoattractant gradients, these cells rapidly polarize and migrate directionally at speeds that can reach up to 20 μm/min, using anterior pseudopod extension together with posterior contraction and retraction.

Chemotaxis is also essential for the survival of the social amoebae, *Dictyostelium discoideum*. In conditions of nutrient deprivation, this organism enters a program that allows it to survive by differentiating into highly resistant spores. *D. discoideum* cells do so by sensing and migrating toward secreted adenosine 3′, 5′ cyclic monophosphate (cAMP) signals, thereby forming tight aggregates that eventually differentiate into fruiting bodies composed of spores atop stalks of vacuolated cells. Intriguingly, as they are migrating through chemotaxis, these cells align in a head-to-tail fashion and form characteristic streams (Figure 1, part B). The essential role of chemotaxis in this eukaryote provides an excellent model to study the

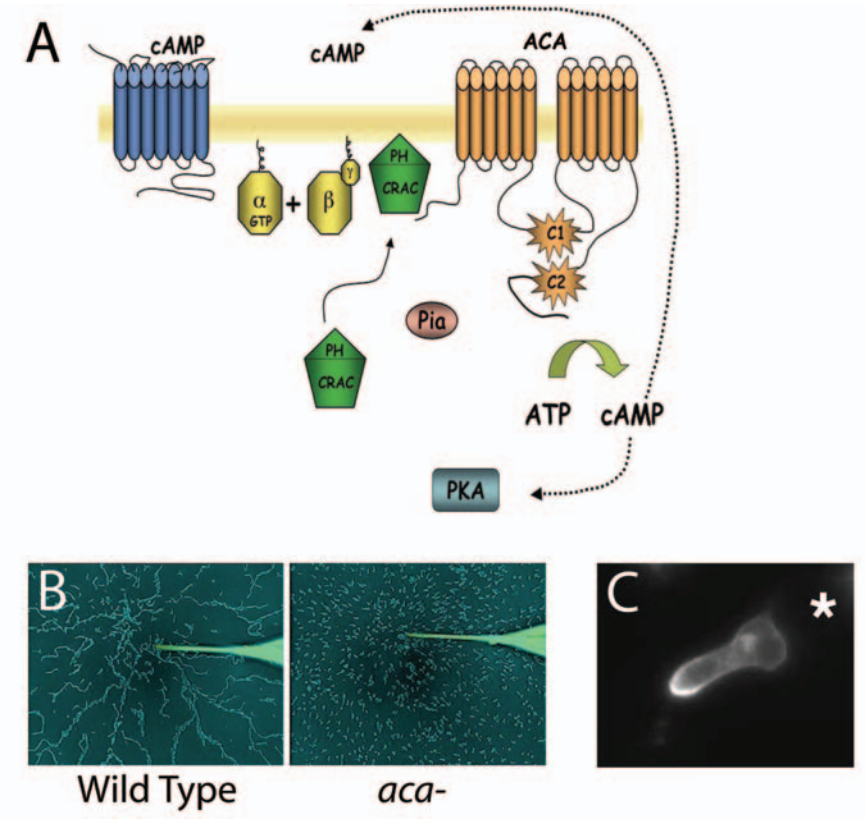


**Figure 1.** Role and regulation of the adenylyl cyclase A (ACA) in *Dictyostelium discoideum*. (*A*) Schematic representation of the signaling pathway regulating the activation of ACA by G protein–coupled receptors. ACA is predicted to be composed of two sets of six transmembrane helices followed by a conserved cytoplasmic loop where the catalytic domain resides. The activation of ACA requires, in addition to the βγ-subunits of heterotrimeric G proteins, two cytoplasmic regulators: (1) the pleckstrin homology (PH) domain-containing protein, cytosolic regulator of adenylyl cyclase (CRAC) and (2) Pianissimo (Pia). (*B*) Images of *aca*⁻ and wild-type cells as they are moving by chemotaxis toward a micropipette containing 1 μM of adenosine 3′, 5′ cyclic monophosphate (cAMP). Wild-type cells align in a head-to-tail fashion and form streams, while *aca*⁻ cells orient individually toward the micropipette. (C) Fluorescent image of *aca*⁻ cells expressing ACA-yellow fluorescent protein (YFP) as they are migrating toward the tip of a micropipette containing 1 μM of cAMP. The star indicates the position of the micropipette. ATP, adenosine triphosphate; GTP, guanosine triphosphate; PKA, protein kinase A. C1 and C2 refer to two catalytic domains of ACA.

biochemical and genetic bases of directed cell migration.

In both leukocytes and *D. discoideum* cells, chemotactic responses are initiated when chemoattractants bind to and activate G protein–coupled receptors (GPCRs), leading to the dissociation of the heterotrimeric G proteins into α and βγ subunits and the activation of a plethora of downstream effectors. Since chemotactic behavior is observed in very shallow attractant gradients, mechanisms that amplify extracellular cues into highly localized intracellular signaling responses must be at play. Indeed, work initially performed in *D. discoideum* established that proteins harboring pleckstrin homology (PH) domains specifically translocate to the leading edge of polarized migrating cells using a pathway involving the lipid products of PI3 kinase. These findings were later observed in leukocytes and led to the hypothesis that PH-domain–containing proteins act as adaptors to spatially nucleate specific signaling events at the leading edge of cells migrating through chemotaxis.

To further understand how signals are transmitted during chemotaxis, we studied the spatial and temporal distribution of the enzyme responsible for the generation of the major chemoattractant in *D. discoideum*, adenylyl cyclase A (ACA). In *D. discoideum*, cAMP binds to a family of GPCRs that activate downstream effectors, including ACA. This leads to the production of cAMP, which is partly secreted to relay chemotactic signals to neighboring cells (Figure 1, part A). We fused ACA to the yellow fluorescent protein (YFP) and studied the cellular distribution of this functional chimera in differentiated migrating cells. Remarkably, we found that the plasma membrane labeling of ACA-YFP is highly enriched at the back of polarized cells (Figure 1, part C). The posterior confinement of ACA-YFP depends on the actin cytoskeleton but bears no relationship to the activity of the essential cytosolic regulator of adenylyl cyclase (CRAC) or the cAMP effector protein kinase A. Indeed, we recently found that CRAC independently regulates chemotaxis and ACA activation (Comer FI *Curr Biol* 15: 134–9, 2005). We also found that, although cells lacking ACA can polarize and migrate to exogenous sources of cAMP, they do not line up head-to-tail and form streams. We therefore propose that the enrichment of ACA at the back of cells migrating through chemotaxis provides a compartment from which cAMP is secreted to locally act as a chemoattractant, thereby allowing cells to follow each other and greatly amplify the chemotactic response. Indeed, we showed that a mutant of ACA exhibiting a dramatically reduced asymmetric distribution does not stream.

As leukocytes are known to secrete a variety of chemoattractants, we propose that the streaming behavior observed in *D. discoideum* is common to higher eukaryotic cells and that the enzymes responsible for chemoattractant synthesis will also be enriched at the posterior of other cells. Moreover, since breast cancer cells and melanoma cell lines have been shown to express and respond to specific subsets of chemokines, it is possible that misregulation of a signal relay pathway is one of the pathological events that lead to carcinoma invasion.

■ **Carole A. Parent, PhD**
Principal Investigator
Laboratory of Cellular and Molecular Biology
NCI-Bethesda, Bldg. 37/Rm. 2066
Tel: 301-435-3701
Fax: 301-496-8479
parentc@helix.nih.gov

# Leadership Changes and the Path Forward

As Dr. von Eschenbach has announced, I have accepted his offer to assume the directorship of the CCR. I am looking forward to this new challenge and am committed to working with the CCR community to continue making great strides in cancer and AIDS research.

The formation of the CCR in 2001 has generated a highly integrated infrastructure that is tremendously successful in moving discoveries at the laboratory bench to the clinical setting. High-impact clinical studies and major advances in biomedical technology within the division have contributed significantly to taking the lessons learned at the bedside back to the laboratory bench. In order to more efficiently provide vision and direction for both the clinical and basic research programs in the CCR, the NCI leadership has chosen to appoint two scientific directors. Lee Helman, MD, is serving as acting scientific director for clinical research, while I am serving as acting director for the basic sciences. Lee has a strong vision for and commitment to the CCR clinical research enterprise and will invite the participation of thought leaders both within the CCR as well as from outside as he looks for opportunities to further increase the impact of the clinical program. Lee and I are committed to working diligently together to continue implementing the Reengineering of the NCI's Intramural Research Program that was initiated shortly after Dr. von Eschenbach's arrival at NCI. This process will ensure that the CCR continues to make the highest basic science impact; lead in the development, application, and export of new technologies; translate novel findings into the clinical setting; and provide leadership in the conduct of science-based early phase clinical trials.

Earlier this year, I met in small groups with all of the Lab and Branch chiefs to discuss a number of important issues facing the CCR. The input and suggestions made during these meetings were highly valuable in making me aware of many issues important to our research community, including increased communication with the CCR leadership, timely dissemination of Laboratory and Branch budget information, requests that the CCR priority areas be articulated, thoughts about our organizational structure, and ideas for further increasing the visibility and scientific impact of the CCR.

As a result of these Lab and Branch Chief meetings, several actions have been taken, and several other action items were identified. The first, and perhaps most important, is that the Intramural Advisory Board (IAB) has been dissolved and the CCR will constitute an Advisory Board to the Director, CCR. The CCR Advisory Board (CAB) members will be representative of the CCR as a whole and will be asked to provide advice as well as help tackle specific challenges being faced by the CCR. Second, there was considerable discussion about moving to what I would refer to as a "hybrid" organizational model for the CCR. The need to maintain many Laboratories and Branches as they currently exist was clearly articulated. At the same time, there was considerable consensus that there are probably some Labs/Branches that could be merged or realigned to create new programs of higher impact in key areas, and as we have articulated in the Reengineering document, there may be other opportunities for developing cross-cutting programs across existing structures. Discussions and planning around this concept will be occurring over the next few months. Finally, there was considerable agreement on the need to begin recruiting new tenure track investigators to further strengthen areas of excellence and exploit new scientific opportunities. Since we will be in a period of flat or reduced budgets in the next few years, we will need to recruit our future leaders thoughtfully and in a manner that will add the greatest value to the CCR as a whole. As we move forward with the scientific vision for CCR, continue our dialogue with the various NCI advisory boards and the NCI leadership, and constitute the CAB, your advice and input will be solicited and valued.

Substantive concerns about the current review and reward structure as it relates to participation on multidisciplinary research teams have been brought to my attention. Since the CCR supports and encourages people from diverse backgrounds to work together to address complex scientific problems, we are committed to ensuring that participation in team science is appropriately reviewed. This issue was one of the agenda items at our joint Board of Scientific Counselors (BSC) meeting in March, and we addressed revising the review criteria to include specific descriptors for team science to more fully encompass the review of team-based research in addition to the well-established expectations for individual research. The issue of review and reward for scientific teams is currently a topic for the entire scientific community, and the CCR is well positioned to provide some leadership in this area. The members of the BSC welcomed this concept. Another major agenda item at this BSC meeting was the role of the site visit team compared with that of the BSC. At this meeting, the role of the site visit team was articulated as the component that focuses on the scientific merit of the science presented, both in written and oral form, and to convey their level of enthusiasm for the science to the BSC committee. The role of the BSC is to consider this expression of scientific merit in the context of existing resources and to make resource recommendations consistent with the future vision of the program.

Although we have entered a period of administrative and resource challenges, we are also at a time of unparalleled scientific opportunity. In that regard, your ideas for defining critical and distinctive scientific opportunities, as well as strategies for redeploying resources in support of the highest priority objectives will be important. I look forward to working with all of you to assure CCR's continued leadership in basic and clinical research.

**Robert H. Wiltrout, PhD**
Director

# From SDS Gels to Supercomputers and More: Highlights of the Symposium Honoring the Scientific Achievements of Dr. Jacob Maizel, Jr.

I still vividly remember the Cray supercomputer (and sitting on the top of it) that our chief, Jacob V. Maizel, Jr., showed to me 15 years ago when I joined the Laboratory of Experimental and Computational Biology (LECB) (formerly the Laboratory of Mathematical Biology)—it was the only one dedicated to biomedical research and was so powerful that, needless to say, I felt excited and proud to be part of the LECB. A few years later, I was reading the *Molecular Biology of the Cell* by Bruce Alberts et al., and what a surprise, Jake was cited as the inventor of SDS gel electrophoresis! (See Second Edition, 1989, page 173, Table 4-9, "Maizel introduced the use of sodium dodecyl sulfate [SDS] for improving polyacrylamide-gel electrophoresis of proteins.") (See also Maizel JV Jr. SDS polyacrylamide gel electrophoresis. *Trends Biochem Sci* 25: 590–2, 2000.)

How are SDS gels related to supercomputers? The answer to this and other questions about the diverse, exciting, and profoundly influential life of Jake Maizel as a scientist was provided by prominent researchers gathered at the symposium, *SDS-PAGE, Genes, and Supercomputers*, organized by Robert Blumenthal to honor Jake's scientific achievements, show our gratitude to him, and somewhat soften our sadness that he is leaving our lab.

In his welcoming remarks to the more than 100 guests, Robert Wiltrout, Director of the CCR, described Jake's long and productive scientific career and emphasized some of his seminal discoveries, including the first observation of the cleavage of large viral precursor molecules to functional proteins. After Jake obtained his PhD in biochemistry in 1959 from Caltech in Pasadena, CA, he briefly joined the NIH as a scientist in the Laboratory of Cell Biology, then became a Professor in the Department of Cell Biology at Albert Einstein College of Medicine in Bronx, NY. In 1974, he returned to the NIH, initially as Head of the Molecular Structure Section of the National Institute of Child Health and Human Development, and from 1984 until his retirement had been Chief of the LECB. His early work was on the identification and characterization of poliovirus and adenovirus structural proteins, mostly by using the SDS gels he invented. In the mid-1970s, he developed an interest in the genes encoding those proteins and also in RNA structure and function (particularly as predicted by computer-assisted analysis) and how RNA can be visualized by electron microscopy. When rapid nucleic acid sequence determination techniques appeared in the late 1970s, he began to apply computers in the analysis of biological sequences. This led to the realization that the biomedical field was ready for the application of supercomputing and to the development of a facility known as the Advanced Biomedical Computing Center to encourage maximum scientific utilization of this technology.

Jacob V. Maizel, Jr., PhD

After the welcoming remarks and introduction of the speakers by Robert Blumenthal, Wolfgang K. (Bill) Joklik emphasized how enormously the invention of SDS gel electrophoresis by Jake has increased the pace of scientific research by giving investigators the ability to separate proteins and determine their size. William Studier reflected on how the SDS gel methodology was critical for understanding *T7* gene expression control. This understanding resulted in the development of the T7-based inducible protein expression system and the recent improvement involving autoinduction of protein expression, where the target protein expression can reach more than half of the total cell protein.

George Vande Woude, who has known Jake for more than 40 years, remembered how he and Jake analyzed the similarity between mouse genomic DNA-containing sequences (sarc) and the acquired cell sequences (src) of Moloney sarcoma virus (*Science* 207: 1222–4, 1980). Others noted additional accomplishments of Jake's, including his seminal discovery of O-linked glycosylation of proteins in the cytoplasm (*Virology* 58: 345–61, 1974), which has had a profound influence on glycobiology.

Bill Joklik said that Jake is one of those few scientists who greatly influenced the work of others, and this became increasingly clear as others spoke.

Charles DeLisi remembered how Jake told him many years ago that there is a way to do whole genomic sequencing, and how this remark affected him later when initiating the human genome project. Matija Peterlin commented that without Jake, he would not have gone into science. Matthew Scharff said that he is most of all indebted to Jake for helping him become a scientist. All of the speakers, who also included William Chin, Ellie Ehrenfeld, Robert Jernigan, Joe Kates, Robert Lenk, David Lipman, Pradman Quasba, and Bruce Shapiro, spoke fondly of Jake—they all, without exception, expressed their love and appreciation for him.

Jake thanked all of the guests for this touching symposium and modestly, as always, concluded that he has only helped other scientists to make discoveries. Although we are all saddened by his departure, we wish Jake much happiness in his new career as a *sea captain!* —Bon voyage!

■ **Dimiter S. Dimitrov, PhD**

## ■ TRANSLATIONAL RESEARCH

# A Novel HIF-1α–Myc Pathway Regulating Hypoxia-induced Cell-cycle Arrest

Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, and Huang LE. HIF-1alpha induces cell cycle arrest by functionally counteracting Myc. *EMBO J* 23: 1949–56, 2004.

Solid tumors harbor hypoxic regions that are not only critical for tumor development and progression, but are also associated with resistance to chemotherapy and radiation therapy. Hypoxia-inducible factor 1α (HIF-1α), a basic helix-loop-helix (bHLH) transcription factor of the PAS protein family, plays an essential role in the transcriptional activation of genes involved in angiogenesis and glycolysis, which are required for tumor development and progression. In many human cancers, HIF-1α and HIF-2α, a close member of the HIF-α family, are overexpressed, and their expression levels are correlated with the degree of malignancy. Moreover, genetic studies have shown that *Hif1α*-null tumors grow much slower in a poor vascular environment, as compared with their *Hif1α* wild-type counterparts.

HIF-1α expression is regulated primarily by posttranslational stabilization, resulting from inhibition of the ubiquitin-proteasome pathway that targets the oxygen-dependent degradation domain (ODD) of HIF-1α. HIF prolyl 4-hydroxylases function as oxygen sensors to modify two proline residues within the ODD, thereby enabling the VHL E3 ubiquitin ligase to bind specifically to the hydroxyprolines for HIF-1α polyubiquitination. Accordingly, deletion of the ODD renders HIF-1α stable and capable of binding the hypoxia-responsive element (HRE) and activating the downstream target genes.

Interestingly, apart from stimulation of angiogenesis and glycolysis for cell proliferation and survival, hypoxia also induces cell-cycle arrest, apparently against tumor development. Although the results from *Hif1α*-null cells indicate that HIF-1α is required for hypoxia-induced upregulation of $p21^{cip1}$, a key cyclin-dependent kinase inhibitor that controls the $G_1$ checkpoint, the role of HIF-1α in the cell cycle remained controversial. Moreover, it remained obscure how HIF-1α transcriptionally activates $p21^{cip1}$ due to the lack of HIF-1α–bound HRE in the promoter.

*…we validated that the N-terminal HIF-1α is critical for cell-cycle arrest, indicating a novel mechanism for HIF-1α function.*

To provide direct evidence that HIF-1α controls the cell cycle, we took advantage of an ODD-deficient HIF-1α and demonstrated that expression of HIF-1α in normoxia is sufficient to induce $G_1$ arrest. As expected, HIF-1α activates $p21^{cip1}$ expression, and conversely, HIF-1α–induced cell-cycle arrest is $p21^{cip1}$ dependent. Therefore, HIF-1α induces $G_1$ arrest via the activation of $p21^{cip1}$.

To understand the mechanism underlying $p21^{cip1}$ activation in hypoxia, we created two functional mutations that inactivate HIF-1α DNA-binding and transcriptional activation, respectively. To our surprise, both mutants were still able to activate $p21^{cip1}$ and to cause $G_1$ arrest, despite their inability to upregulate known HIF-1α target genes, such as *VEGF*. Thus, neither HIF-1 transcriptional activity nor its DNA binding is required for $p21^{cip1}$ activation, implying a novel HIF-1α function in regulating gene expression.

In pursuit of the distinct function of HIF-1α, we hypothesized that HIF-1α upregulates $p21^{cip1}$ by virtue of functionally counteracting Myc, a known repressor that binds the transcription activator Miz-1 of $p21^{cip1}$. Consistently, hypoxic treatment or HIF-1α expression in normoxia overrode Myc-targeted gene expression; hypoxia/HIF-1α not only upregulated Myc-repressed gene $p21^{cip1}$, but also downregulated Myc-activated genes, such as *TERT* and *BRCA1*. RNA silencing experiments demonstrated

a critical role for Myc in HIF-1α action. Moreover, chromatin immunoprecipitation analysis of the *p21*$^{cip1}$ promoter showed that Myc binding was markedly weakened by hypoxia or HIF-1α, suggesting that the HIF-1α action is mediated by the displacement of Myc from the *p21*$^{cip1}$ promoter.

We showed further that HIF-1α forms a weak complex with Myc. The protein-protein interaction is mediated by an HIF-1α N-terminal region consisting of bHLH and PAS domains. Finally, we validated that the N-terminal HIF-1α is critical for cell-cycle arrest, indicating a novel mechanism for HIF-1α function.

This study has demonstrated that HIF-1α employs at least two mechanisms for regulating gene expression: in addition to the classical mode of action involving binding to the HRE plus transactivation via transcriptional activation domains, HIF-1α functionally antagonizes Myc via its N-terminal region to override the expression of Myc-targeted genes that lack a canonical HRE. This new pathway may signify a new set of hypoxia-responsive genes that cannot be accounted for by the previously identified mechanisms. In addition, the independent action of HIF-1α N-terminal indicates that HIF-1α polypeptide, devoid of transactivation domains and DNA binding activity, is in fact functional; HIF-1α mutants in this nature are still able to regulate hypoxia-responsive genes that lack HREs. Therefore, the interpretation of effects of such "dominant-negative" mutants may need to be reevaluated.


- **Minori Koshiji, MD, PhD**
  Visiting Fellow
  koshijim@mail.nih.gov
- **L. Eric Huang, MD, PhD**
  Principal Investigator
  Laboratory of Human Carcinogenesis
  NCI-Bethesda, Bldg. 37/Rm. 3044B
  Tel: 301-402-8785
  Fax: 301-480-1264
  huange@mail.nih.gov


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